Directed Differentiation of Embryonic Stem Cells to Cardiomyocytes in a Bioreactor

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Introduction

Heart diseases are top ranked as causes of morbidity and mortality in the US and most developed countries[1-3 4 5]. Most heart maladies are associated with the death of significant portion of cardiomyocytes leading to myocardial infarction/heart attacks and permanently impaired heart function.

Presently, the only therapeutic modality that effectively reconstitutes the heart function is heart transplantation. However, its widespread utility is hindered due to the scarcity of donor organs and complications associated with immune suppressive regimens. Hence, the search for alternative sources of heart cells including embryonic stem cells (ESCs) has intensified in recent years. ESCs are pluripotent, i.e. they are able to differentiate into all cell types of the body. More importantly, ESCs have an unlimited proliferation capacity if cultured under certain conditions and hence can serve as inexhaustible source of cardiomyocytes. Transplantation studies have shown that cardiomyocytes from differentiated ESCs have the potential to integrate with the damaged heart[6-10]. A major challenge however has been to reproducibly and efficiently generate a pure population of cardiomyocytes from ESCs.

We are developing methodologies for more efficient cardiac differentiation of embryonic stem cells, and subsequently scalable systems for their production in large quantities as required for clinical applications. We have used mouse ESCs (mESCs) as a model system and translated important findings to human ESCs (hESCs) because of the physiological similarities between the two species. Mouse ESCs were selected as a model system because (1) of their relatively high proliferation rate which enables more experiments to be done in a shorter time, and (2) of their relatively cheaper culture cost. We have efforts to better understand customary cardiac differentiation methods and to identify vital culture parameters to improve the outcome of the differentiation. Subsequently, we determined conditions that optimize the differentiation. The current differentiation methods have major limitations such as highly varied outcome[11, 12] and low yield of cardiomyocytes[6, 8, 11, 13, 14]. The differentiation methods rely on serum for nutrition and differentiation cues. The exact composition of serum is undefined but it appears to provide a mixture of proteins, hormones and other factors that support the growth of cells. It is speculated that the some of these growth factors promote cardiac differentiation, however serum-based differentiation generates a highly heterogeneous differentiated cell population with low percentage of cardiomyocytes. It is has also been difficult to separate the cardiomyocytes from the rest of the population. The differentiation outcome is also greatly variable with each batch of serum due to unknown differences in growth factor content. But studies for isolating factors that can

yield a pure cardiomyocyte population have been hindered because of dependence on serum for nutrition. The signals originating from the serum interfere with agonists we attempt to study by inhibiting, suppressing or synergizing their effect. Therefore, there have been extensive efforts to develop a defined serum-free growth medium (DSFM) which can propagate cell culture without affecting differentiation. This can allow for studies to understand processes that govern ESC differentiation and subsequently to develop agonists and conditions that can control these processes to obtain the desired differentiation outcome, i.e. a pure population of cardiomyocytes. Another drawback of serum-based culture is that these cells are not fit for transplantation. Cells cultured in serum can pass on pathogens from its animal source, can trigger foreign body reactions and can cause rejection of transplanted cells due presence of animal proteins. Using a DSFM can also reduce such a risk.

We utilized a DSFM prepared with Knockout Serum Replacer (KSR, Invitrogen, Carlsbad, CA) because it supports well ESC culture and differentiation[15]. Apart from the currently used non-specific cardiomyocyte stimulants such as DMSO and Retinoic Acid (RA)[16, 17],we investigated physiologically relevant growth factors to direct cardiac differentiation. Studies on embryonic heart development have shown that bone morphogenetic protein (BMP) signaling has a direct role in cardiac induction and also terminal differentiation of cardiac tissue[18, 19]. We treated mESCs exogenously with BMP-4 to initiate this signaling. Our results indicate that BMP-4 successfully directs cardiac differentiation of mESCs and the stimulant RA was ineffective under DSFM conditions.

Conventional ESC culture is carried out on dishes which are not optimal for cell production in a clinically relevant scale. Stirred suspension bioreactors promote three dimensional non-adherent cell culture and therefore have potential for maximal scale up of cell production. We developed a method for directing cardiac differentiation of mESCs with BMP-4 in bioreactors without serum.

Our current efforts are focused on translating these findings to hESC differentiation aiming at the development of a scalable system for the generation of cardiomyocytes for cell therapies. Concurrently experiments with mESCs are yielding data to further our knowledge of ESC specification for designing differentiation protocols characterized by high efficiency.

Results:

BMP-4 directed differentiation in mESCs

Under defined serum-free conditions (DSFM), mESCs if untreated with stimulants tend to differentiate towards neural-like cells. Onset of such ectodermal differentiation is characteristic of invitro ESC differentiation in the absence of any influencial signals. Treating mESCs in DSFM with BMP-4 did not reveal any neural cells indicating that ectodermal and mesodermal differentiation are separate routes that ESCs can commit to in vitro. Additionally we observed beating cells which demonstrates

that BMP-4 initiates signaling that leads to cardiomyocytes. This is in accordance with previous studies that show that BMPs have a direct role in cardiac induction and differentiation during embryonic cardiogenesis [18, 19].

We characterized the beating cell clusters obtained by BMP-4 treatment in DSFM and compared the results with serum-based differentiation. Unlike the serum-based differentiation, the curve representing the percentage of beating clusters with time of mESCs treated with BMP-4 was reproducible. The first appearance of beating was day 10 and the curve reached a maximum by days 14-15 consistently. The beating percentage was also consistently higher – approximately 90% of EBs were beating (Figure 1)- compared to the serum-treated counterparts.

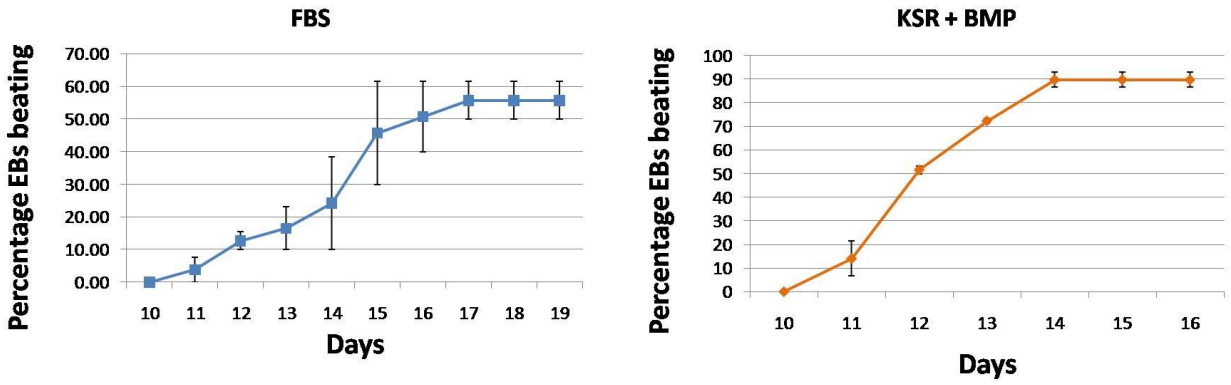


Figure 1: Comparison of beating curves between FBS-based and BMP-based differentiation.

Reverse transcription-PCR (RT-PCR) shows that the BMP treatment caused upregulation of cardiomyocyte-specific genes such as α-myosin heavy chain (α-*MHC*), *GATA-4*, *Nkx2.5*, and *ANF* compared to the BMP untreated control. The expression of α-MHC was much higher in the BMP- than the serum-based differentiation. This is expected because of the higher percentage of beating clusters in the BMP differentiation. Likewise, the BMP untreated KSR controls expressed minimal α-MHC (Figure 2).

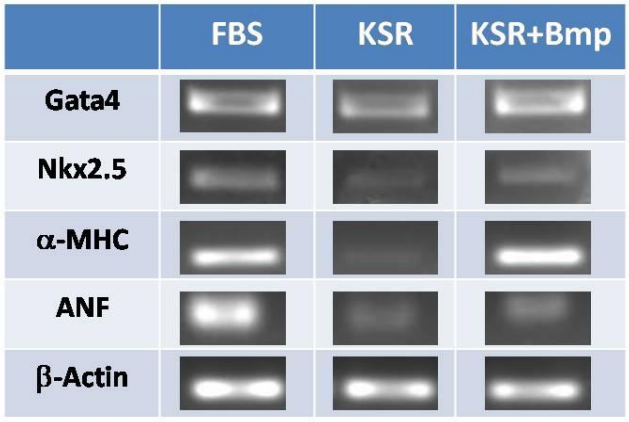


Figure 2: RT-PCR data for cardiomyocyte genes for serum, DSFM (KSR) and BMP + DSFM conditions

Addition of the BMP antagonist Noggin inhibited the ESC differentiation into cardiomyocytes. Noggin-treated cells did not show any beating despite BMP treatment. Quantitative PCR (qPCR) showed a down-regulation of cardiomyocyte genes *MHC* and *Nkx2.5* in noggin-treated cells when compared to BMP-treated cells and similar to BMP untreated control. These results confirm that BMP signaling induces cardiac differentiation. Inhibition of BMP signaling causes non-specific differentiation with only a small percentage of cells becoming cardiac cells.

Scale up of BMP-directed differentiation in a bioreactor

We explored the feasibility of scaling up BMP-based directed differentiation of mESCs cultured in dishes to continuously stirred bioreactors. First, we asked if the ESC differentiation is affected by the hydrodynamic conditions in the bioreactor. Mechanical cues stemming from different environment can affect ESC differentiation and could also alter the action of BMPs. Therefore, we compared differentiated cells obtained from dish and bioreactor cultures. Beating cells obtained from the bioreactor upregulated cardiomyocyte markers in a similar fashion as assessed by qPCR and immunostaining.

A reproducible curve depicting the percentage of beating cell clusters vs. time for bioreactor cultures was obtained and was similar to that for static cultures. In both cases, the first beating clusters appeared on day 10 and the maximum beating percentage was reached by day 14. The maximum beating percentage was also similar - approximately 90% EBs beating.

Next, we plan to compare the cell proliferation and cardiomyocyte yield between dish and bioreactor cultures.

Studies on human embryonic stem cells (hESCs)

The understanding of mESC differentiation to cardiomyocytes under defined culture conditions provides clues for the differentiation of hESCs aiming to generate cardiomyocytes for cell therapies. To successfully translate mESCs differentiation methodologies to hESCs differentiation, we first attempted to differentiate hESCs in FBS-containing medium (similar to the mESC protocol).

hESCs differentiation to cardiomyocytes was initiated by forming EB in non adherent (Petri) dishes (Figure 3b). Beating cell clusters were first identified at

differentiation day 10 and the percentage of beating cells was usually increased with time.

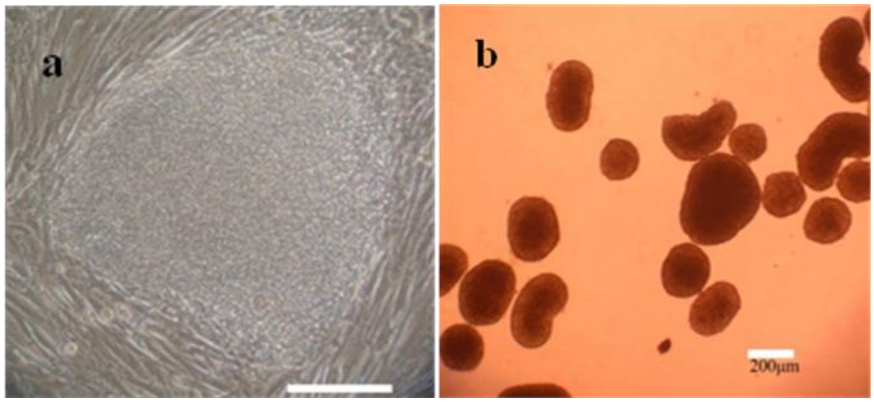
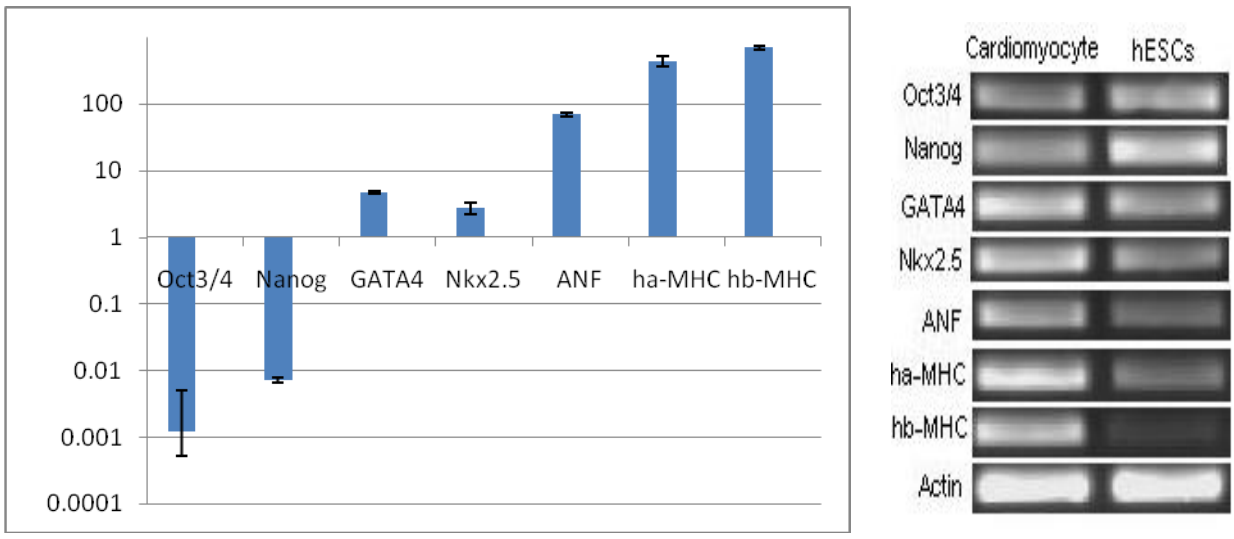


Figure 3: Differentiation of hESCs to cardiomyocytes. (a) The morphology of hESCs maintained on MEF (Bar=250μm). (b)EBs in suspension culture (Bar=200μm).

Quantitative PCR (Figure 4 Upper Panel) revealed an increased expression of heart cell-specific genes in differentiating hESCs compared to undifferentiated. These genes include the atrial natriuretic factor (ANF), human α-myosin heavy chain (hα-MHC) and human β-Myosin Heavy Chain (hβ-MHC). The expression of cardiac markers NKX2.5 and α-actinin were also identified by immunofluorescence (Figure 4, lower panel).



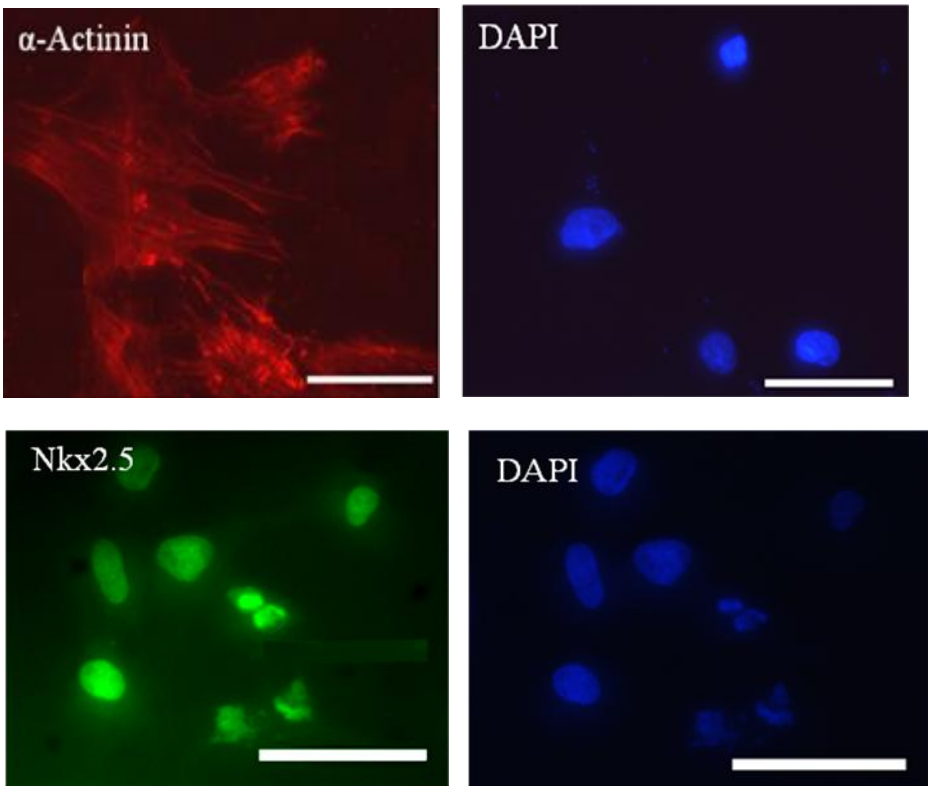


Figure 4: Cardiac marker analysis of cardiomyocytes. Upper Panel: RT-PCR analysis of Oct4, Nanog, GATA4, Nkx2.5, ANF, hα-MHC and hβ-MHC in undifferentiated and differentiated cells. Lower Panel: After seeing cells contracting, they were dissociated as single cells to culture for an additional 3 days and assessed for α-Actinin and Nkx2.5 immunoreactivity. DAPI was used for staining nuclei. Bar=50 µm.

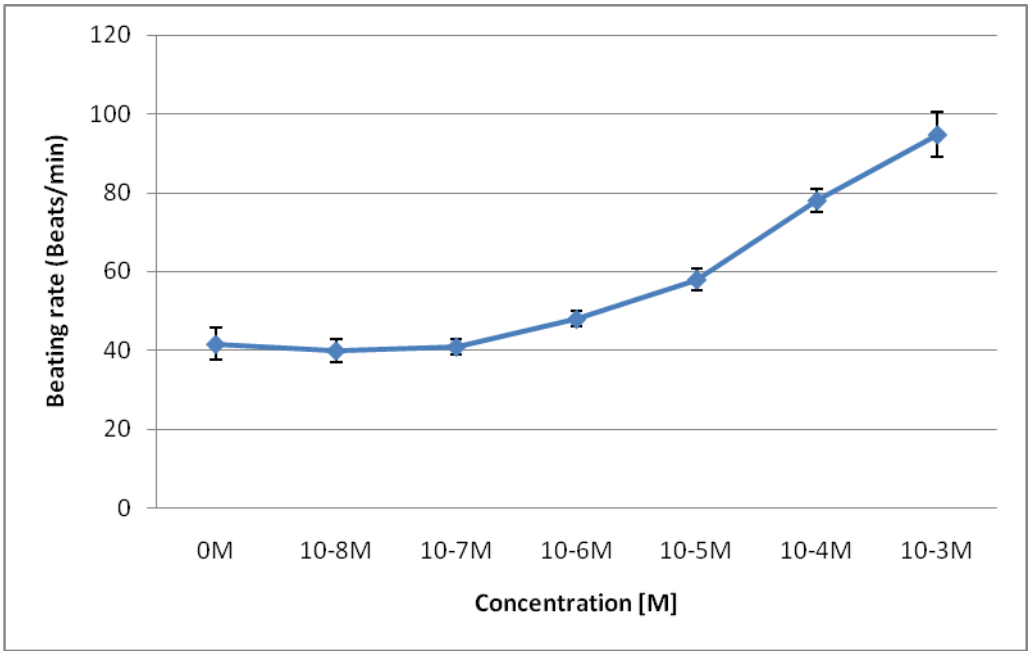


Figure 5: Effect of IBMX (an inhibitor of phosphodiesterases) on the beating rate of hESCs derived cardiomyocytes.

Furthermore, we assessed the functional characteristics of cardiomyocytes compared to native heart muscle cells. Stimulation by isobutyl methylxanthine (IBMX, an inhibitor of phosphodiesterase) resulted in a dose-dependent increased contraction rate (Figure 5). These results indicate the presence of a cAMP-dependent mechanism that mediated the contracting of cells.

Although our hESCs-derived cardiomyocytes exhibit characteristics of heart muscle cells, the current efficiency of differentiation is very low. Thus, our efforts are focused on developing a protocol for differentiation of hESCs to heart muscle cells by growth factors (BMP, Activin) in a directed way.

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